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Antifouling potential of symbiotic marine bacterium *Bacillus subtilis* MUT:M15 associated with the cuttlefish *Sepia* sp.

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The antagonistic marine bacterium *Bacillus subtilis* MUT:M15 isolated from the surface of *Sepia* sp. was identified using 16S rRNA gene sequencing. Intracellular and extracellular extracts of this bacterium was screened for antibacterial and antifouling activities. The results showed that both intracellular and extracellular extracts substantially reduced the growth and attachment of biofilm-forming bacterium, *Alteromonas* sp. The antifouling coatings (ICE15M and ECE15M) prepared with the intracellular and extracellular extracts of *Bacillus subtilis* MUT:M15 showed that these coatings significantly reduced the settlement of microfouling (bacteria and microalgae) and macrofouling organisms (barnacle, tubeworm, bivalve and gastropod) on the test panels submerged in the seawater. The bioactive fractions isolated from both intracellular and extracellular extracts of *Bacillus subtilis* MUT:M15 using thin layer chromatography were identified as phthalate derivatives by gas chromatography and mass spectroscopy.

[Keywords: Marine natural products; Antagonistic bacteria; Antifouling coating; Antifouling activity; Phthalate derivatives; *Bacillus subtilis*]

Introduction

Biofouling is caused by the settlement of marine organisms such as invertebrates, seaweeds and microbes on the structures submerged into the marine environment[1-3]. Heavy metal compounds such as tributyltin (TBT) oxide, copper, lead, mercury and arsenic were incorporated into the paints to prevent biofouling[4,5]. TBT was one of the well known effective antifouling compound against biofouling, but also more toxic to non-target organisms due to deleterious ill-effects[6,7]. As most of the compounds currently used for antifouling purpose are causing pollution to the environment, there is a need for non-toxic (eco-friendly) compounds for biofouling management[8]. Natural products from different organisms are suggested as potential alternative to toxic biocides[9,10], since they are effective, less toxic and biodegradable[11,12]. Some of the bioactive natural products such as terpenoids, steroids, carotenoids, phenolics, furanones, alkaloids, peptides and lactones isolated from wide range of organisms have been documented as natural product antifoulants[13,14]. Some of the natural product based antifouling coatings are commercialized which include Sea Nine – 211, Netsafe and Pearlsafe[15,16].

Naturally, products obtained from marine organisms have unique structures compared with terrestrial organisms[17,18] and are having potential application in pharmaceutical and industrial fields[19]. Especially, marine bacteria capable of producing bioactive compounds are commonly associated with marine plants and invertebrates[20-22]. Such bacteria have been reported as high percentage of bioactive compounds producers [23,24], which has the ability to repel or inhibit the settlement of marine biofoulers[25,26]. Many previous studies are available in the literature regarding the antifouling activity of surface associated marine bacteria[27-29]. Therefore, aim of the present study was to investigate the antifouling potential of the extracts of symbiotic bacteria associated with the cuttlefish *Sepia* sp. Antifouling coatings were developed by incorporating bacterial extract into epoxy primer and their antifouling activity was evaluated in coastal waters. Results observed in this study may improve our understanding on antifouling activities of marine microbes associated with macroorganisms.

Materials and Methods

Isolation and screening of the bacteria associated with the cuttlefish Sepia sp.

The *Sepia* sp. commonly known as cuttlefish (Phylum: Mollusca) was collected with the help of local fisherman during November 2011 from west

coast of India and kept in a sterile plastic bags containing sterile seawater. Following this, the bags were placed into an ice container and brought to the laboratory for the isolation of surface associated bacteria. The symbiotic bacteria associated with the *Sepia* sp. were scraped off using a sterile nylon brush and suspended in 1ml filter-sterilized seawater. This suspension was serially diluted and spread on Zobell marine agar (ZMA) plates (Himedia, India) for the development of bacterial colonies. The developed colonies were then grown (for 24 h at 37 °C) separately on ZMA plate and a soft agar medium (0.6% of agar added with Zobell Marine Broth and 10% of 24 h old culture of biofilm-forming bacterium *Alteromonas* sp. which was isolated from the acrylic panel submerged in the coastal waters by Satheesh et al.[30] was poured over the developed symbiotic bacterial colonies. After 24 h of incubation, the antagonistic effect was observed with the zone formation around the symbiotic bacterial colony[31].

Identification of the active bacteria

The bacterial colony which showed antagonistic activity were initially characterized based on morphological and standard biochemical methods such as Gram staining, motility, catalase and oxidase activity, gelatin and strach hydrolysis, nitrate reduction, citrate utilization, MR-VP, carbohydrate utilization, indol, H_2S and gas production. Secondly, the overnight grown bacterial culture was centrifuged and the collected cell pellet was added with adequate amount of phenol chloroform for the extraction of DNA. Following this, the extracted DNA was amplified by polymerase chain reaction (PCR) according to the methods described previously by Viju et al.[32]. The phylogenetic tree was constructed with 16S rRNA sequence of others obtained from the NCBI database using neighbor joining method[30].

Isolation of intracellular and extracellular extracts of antagonistic bacteria and assessment of antibacterial activity

The isolated antagonistic bacterial culture was grown in a 1000 ml conical flask containing Zobell Marine Broth (ZMB) for a period of 5 days at 37 °C. Following this, the cell pellet and the supernatant of the culture were partitioned by centrifugation (5000 rpm for 25 min) and mixed with adequate amount of organic solvents. For the extraction of intracellular extracts, 3 ml of methanol was added with 1 g pellet, whereas equal amount (1:1 ratio) of supernatant was added with ethylacetate for the extraction of extracellular extracts (ECE) and placed on a shaker for 72 h to provide proper agitation. The solvent (phase) added with the cell pellet was separated by centrifugation (5000 rpm, 15 min), whereas the solvent (phase) added with supernatant was separated by soxhlet extractor. Following this, the extracts (intracellular and extracellular) present in the solvent phase were isolated by evaporating the solvent with an evaporator and their bioactivity was tested using the agar disc diffusion method. For this, sterile disc (6 mm, Himedia, India) loaded with bacterial extract (prepared by dissolving 20 mg of extract in 1 ml of DMSO) was placed onto Mueller-Hinton agar (Himedia) plates seeded with biofilm-forming bacteria (*Alteromonas* sp., *Pseudomonas* sp. and *Gallionella* sp.). The plates were then incubated at 37 °C for 24 h and the antibacterial activity was measured in the zone appeared around the bacterial colony[33].

Antibiofilm activity—ELIZA Method

The bacterial extract (20 µl) was added (in replicates, n=3) into a well of a polystyrene microtiter plate containing 200 µl of biofilm-forming bacterial (*Alteromonas* sp.) culture broth. While 20 µl of DMSO was added instead of bacterial extract as control for the cross-reference and the plate was incubated at 37 ºC for biofilm formation. After 24 h, the plate was taken out and turned inversely and washed with (0.2 ml) PBS to remove unattached bacteria. After that, 2% of sodium acetate was added to fix the biofilm formed on the wells and the fixed biofilm was stained with 0.1% w v^{-1} crystal violet (0.1% w v^{-1}). Finally, optical density (OD) of the biofilm formed on the wells was determined by a micro ELISA auto reader at a wavelength of 570 nm[34].

Antibiofilm assay—Petri dish method

The antagonistic bacterial extract (0.5 ml) was taken in a sterilized petri dish containing 10 ml of 24 h old biofilm-forming bacterium *Alteromonas* sp. culture and sealed with parafilm. For the control, 0.5 ml of DMSO was added instead of bacterial extract. The content of the dish was mixed properly by moving the dish front and back and the dish was incubated for 72 h at 37 °C. After incubation, the content of the dish was decanted and washed with

PBS buffer to remove the unattached bacteria and was allowed to dry. The dried dish was then stained with 0.1% crystal violet solution and excess stain was removed using distilled water. The remaining adhered stain was destained with 33% acetic acid by placing the dish on a rotary shaker for 15 min and the absorbance of the crystal violet was measured in a spectrophotometer set at 595 nm and repeated (n=3) for the confirmation of the obtained result. The inhibition of biofilm formation was measured using the formula,

$$\text{Biofilm Inhibition (\%)} = \frac{\text{Control OD - Experiment OD}}{\text{Control OD}} \times 100$$

Bioactivity of antifouling coat developed with bacterial extracts

Antifouling coatings were prepared by incorporating the bacterial extract into the epoxy primer along with epoxy resin to assess the antifouling activity of the bacterial extract. In brief, 3 ml of epoxy primer (Gempex) was added with 1 ml of epoxy resin, 1 ml of hardener and 1 ml of bacterial extract (20 mg of bacterial extracts isolated from the antagonistic bacteria were dissolved in 1 ml of DMSO). The control coating was also prepared by adding 1 ml of DMSO instead of bacterial extract. Besides, bioactivity of the coatings was tested using agar well diffusion method. In brief, a well made on the Zabell Marine Agar plate swabbed with biofilm-forming bacterium *Alteromonas* sp. using a well cutter was filled with 100 µl of antifouling coatings and the plate was then incubated at 37 °C for 48 h. After incubation, plate was taken out and observed for zone of inhibition appeared around the wells[32].

Preparation of panels coated with antifouling coating

Antifouling and control coatings were coated on fiber panels (18 × 12 cm) using nylon brush and placed in a sterile chamber for a week for drying. This coating was repeated two more times to increase the thickness of the coating on the panel and all these panels were dried properly.

Anti-microfouling effect of coatings

This experiment was carried out to study the influence of antifouling coating on the settlement of microfouling organisms. The panels coated with antifouling and control coatings were fitted on an iron frame firmly and submerged into the coastal waters (Colachel coast, West coast of India, Latitude-$8^0$17'N: Longitude-$77^0$26'E) at a depth of about 3 m from the mean sea level for a period of five days (05/07/2015 to 09/07/2015). The panels were retrieved after five days of submersion and the settlement of microfouling organisms such as bacteria and microalgae were estimated. For this, a microbial suspension was prepared by scrubbing off the microbial cells adhered on the coatings and serially diluted in 1ml of filter-sterilized seawater. For the enumeration of bacteria, a small (200 µl) volume of microbial suspension was spread on ZMA plate and incubated at room temperature. After 24 h of incubation, the bacterial colonies developed on the plate were counted with a microbial colony counter (LAPIZ, Medica). While a small amount of microbial suspension was also placed on a hemocytometer to estimate the micro algal density. The number of microalgae was counted under the microscope (COSLAB) and the cell density was calculated using the formula,

$$\text{Algal cell density} = \frac{\text{Numbrt of cells counted}}{\text{Number of squares counted}} \times \text{Total number of squares}$$

Anti-macrofouling effect of coatings

This experiment was designed to assess the effect of antifouling coatings against the settlement of macrofouling organisms. The panels coated with antifouling and control coatings were fitted on an iron frame firmly and submerged into the sea (as indicated above) for a period of 100 days (05/07/2015 to 12/10/2015). The panels were retrieved from the raft after 100 days of submersion and transported to the laboratory in a cool box. In the laboratory, the settled macrofouling organisms such as barnacles, tubeworms, bivalves and gastropods were counted manually after scrapping the organisms from the test panels. Besides, another experiment was conducted to assess the fouling inhibition of coating by the biomass method. For this, panels prepared as above were submerged in seawater and the submerged panels were retrieved at 20-day intervals (i.e., after 20, 40, 60, 80 and 100 days of submersion). The panels were brought to the laboratory and the fouling community recruited on the panels was scraped using a scalpel. The dry weight of the fouling assemblage on the panels was measured after drying by keeping in an oven at 60 ºC for 12 h. The biomass of marine biofoulers recruited on the control and antifouling coatings coated panels was calculated by the formula[32],

$$\text{Biomass of biofoulers (\%)} = (FWFC/IWFC)^{1/N} - 1 \times 100$$

where, IWFC- the initial weight of fouling community; FWFC- the final weight of fouling community; N- the number of measurements taken.

Isolation of bioactive fractions using thin-layer chromatography

The bioactive fraction present in the crude bacterial extract was separated using thin layer chromatography. The bacterial extract was loaded on the activated glass plates pre-coated with silica gel, and the plates were then kept in a TLC chamber containing the mobile phase (methanol, ethylacetate and chloroform in the ratio of 2:3:5) for the mobility of compounds. The plates were removed from the chamber after the solvent reached the end point. The plates were air-dried and kept in a chamber in the presence of iodine crystals for the development of spots (compounds). The spots developed were scraped off in adequate amounts and dissolved in the respective solvents (methanol for the fraction (spot) obtained from intracellular extract and eathylacetate for the fraction (spot) obtained from bacterial extracellular extract). After 15 min of centrifugation, the supernatant was collected and its bioactivity was tested by using the agar disc diffusion method against biofilm-forming bacterium *Alteromonas* sp.

FT-IR analysis of bioactive fractions

The infrared spectrum was recorded in a SHIMADZU FT-IR system. A small quantity of TLC purified fraction was placed on the face of a highly polished KBr salt plate and another KBr plate was positioned on the top to spread the compound as a thin layer. The FT-IR spectra were recorded in a wave number from 400 to 4000 cm^{-1} to find out the presence of the functional groups.

GC-MS analysis of bioactive fractions

The chemical constituents present in the bioactive fraction isolated from the bacterial extracts were analyzed using GC Clarus 500 Perkin Elmer equipped with Elite-5MS fused silica capillary column (length-30 mm; diameter-0.25 mm; thickness-0.25 µm), operating in electron impact mode at 70eV. The carrier gas pure helium (99.999%) was flown at a rate of 1ml/minute and an injection volume of 2 µl (split ratio 10:1). The injector port temperature and an ion source temperature were maintained at 250 °C and 280 ºC, respectively. Whereas the initial oven temperature was set at 110 ºC for 2 min with an increasing rate of 10 ºC/minute and the ending temperature was increased to a isothermal point at 280 °C/9 min. Following this, the electron mass spectra of the injected bioactive fraction was taken at 2 min scan interval. The identification of the compound was based on the comparison of their mass spectra with libraries of digitized mass spectra of known compounds[35].

Statistical analysis

Student's t test was used to assess the influence of bacterial extracts on settlement of bacteria on microtiter plates (antibiofilm assays). Whereas the variation between the settlement of microfouling and macrofouling organisms on the panels coated with control and bacterial extract incorporated coatings was examined by one way ANOVA followed by Dunnet multiple comparison test (*P value <0.05 was considered as significant*).

Results

Identification of antagonistic bacterium

The symbiotic bacterium 15M isolated from the surface of *Sepia* sp. showing strong (2.5 mm zone) antagonistic activity against biofilm-forming bacterium *Alteromonas* sp in the agar overlayer method was initially characterized using morphological and biochemical methods. Results revealed that it was Gram positive, non-motile and rod-shaped bacterium. Moreover, it gave positive results for the biochemical tests, such as indol production, citrate utilization, starch and urea hydrolysis and nitrate reduction. Whereas it was negative for methyl red, voges proskauer, oxidase, casein and gelatin hydrolysis. Based on the obtained data, it was concluded that the bacterium belonged to *Bacillus* sp. The results obtained from 16S rRNA gene sequencing also confirmed the genus. The phylogenetic analysis of 1,512 nucleotide sequences obtained from the DNA fragment of the strain 15M isolated from the surface of *Sepia* showed 99% similarity with *Bacillus subtilis*. The nucleotide sequence data have been deposited at GenBank (GenBank, NCBI) with the accession number KX669626.

Antibacterial activity of antagonistic bacterial extracts

The intracellular and extracellular extracts of antagonistic bacterium *Bacillus subtilis*. MUT:M15 substantially inhibited the growth of biofilm-forming bacteria *Alteromonas* sp., *Pseudomonas* sp. and

Galionella sp. The intracellular extract showed 13 mm zone of inhibition against *Alteromonas* and *Pseudomonas* sp. Also, the extract showed 11 mm against *Gallionella* sp. Whereas the extracellular extract showed 14 mm zone of inhibition against *Alteromonas* sp. and 12 mm zone of inhibition against *Pseudomonas* and *Gallionella* sp. (Fig. 1).

Antibiofilm activity—ELIZA Method

The biofilm formation of *Alteromonas* sp. was considerably reduced on the microtiter plate well due to bacterial extract treatment. The OD value of the biofilm developed on the microtiter plate control well was 0.341±0.012. This value was significantly reduced to 0.237±0.017 (Student's 't' test, t stat=8.44, df=4, $p<0.001$) and 0.233±0.015 (Student's 't' test, t stat=9.36, df=4, $p<0.001$) respectively on the wells treated with intracellular and extracellular extracts of the bacterium *Bacillus subtilis*. MUT:M15 (Fig. 2).

Antibiofilm activity- Petri dish method

The bacterial extracts considerably reduced the biofilm formation of *Alteromonas* sp. on petri dish. The intracellular extract of the bacterium *Bacillus subtilis*. MUT:M15 showed 35.62% of biofilm inhibition, whereas extracellular extract showed 42.48% of biofilm inhibition. The variation on biofilm development between control and extract plate can be seen in Figure 3.

Bioactivity of antifouling coating (Agar well diffusion method)

The bioactivity of antifouling coatings tested against biofilm-forming bacteria *Alteromonas* sp. revealed that the coating named as ICE15M and ECE15M developed by incorporating intracellular and extracellular extract of the bacterium *Bacillus subtilis*. MUT:M15 showed strong activity in the agar well diffusion method. The zone of inhibition appeared

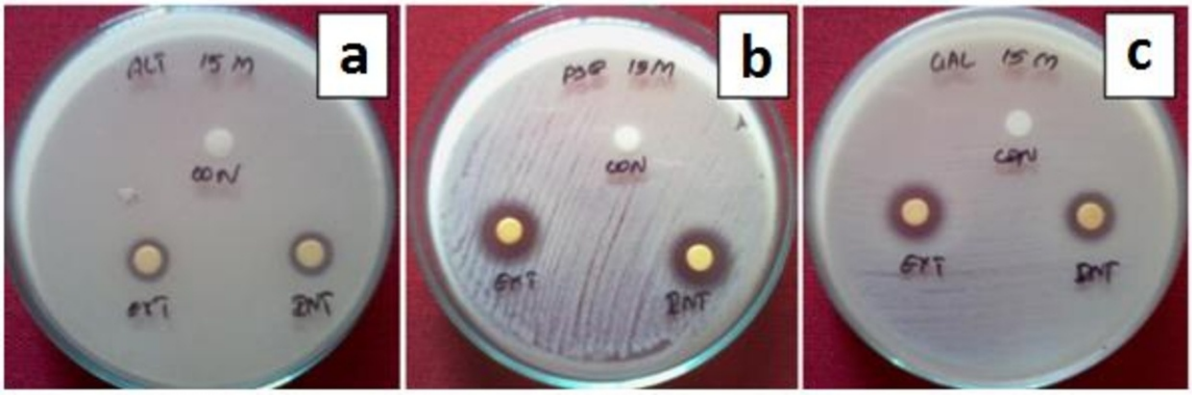

Fig. 1 — Antibacterial activity of intracellular and extracellular extracts of the bacterium *B. subtilis* against biofilm-forming bacteria (a) *Alteromonas* sp. (b) *Pseudomonas* sp. (c) *Gallionella* sp.

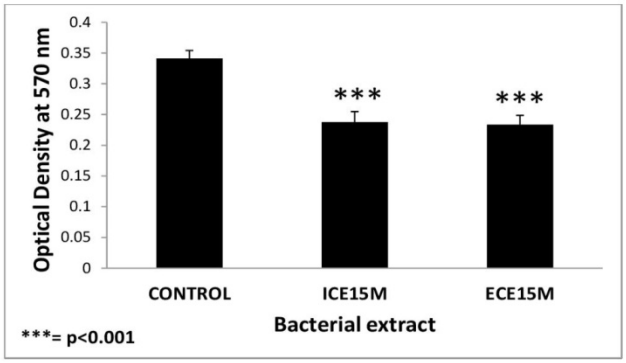

Fig. 2 — Antibiofilm activity of bacterial extracts against biofilm-forming bacterium *Alteromonas* sp. usinng microtitter plate method. The plot showing the difference of biofilm inhibition on control and bacterial extracts treated microtiter wells.

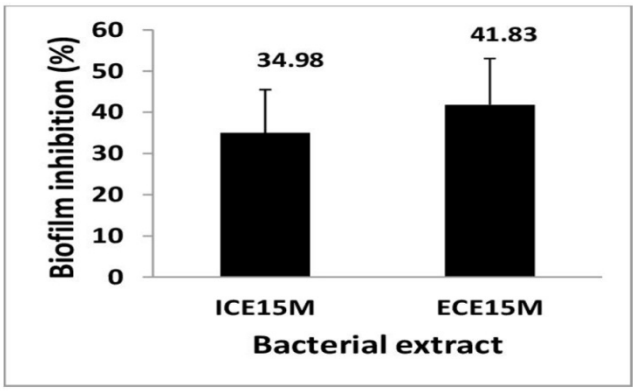

Fig. 3 — Biofilm inhibition of bacterial extracts on Petri dish. The plot showing the biofilm inhibition on control and bacterial extracts treated petri dishes.

arround the wells were 16 and 14 mm, respectively (Fig. 4).

Anti-microfouling effect of coatings

Figure 5 explains the anti-microfouling effect of antifouling coatings. Antifouling coatings (ICE15M and ECE15M) developed with the extracts of *Bacillus subtilis*. MUT:M15 considerably reduced the settlement of microfoulers, such as bacteria and microalgae. The number of bacterial cells adhered on the panel coated with control coating was 288.33±32.53 × 10^6 CFU ml^{-1}, which was reduced to 175±55.67 × 10^6 CFU ml^{-1} and 208±41.93 × 10^6 CFU ml^{-1} on the panels coated with antifouling coatings ICE15M and ECE15M, respectively. One-way ANOVA showed significant variation on the settlement of bacteria between the panels coated with control and antifouling coatings (F=5.160, P<0.04). Similarly, one-way ANOVA showed significant (F=5.233, P<0.04) variation on the settlement of microalgae between the panels coated with control and bacterial extract incorporated antifouling coatings. The number of microalgal cells adhered on the panel coated with control coating was 37.33±6.8 × 10^6 cells ml^{-1}, which was reduced to 18.33±5.7 × 10^6 cells ml^{-1} and 21.66±9.8 × 10^6 cells ml^{-1} on the panels coated with antifouling coatings ICE15M and ECE15M, respectively.

Anti-macrofouling effect of coatings

The anti-macrofouling effects of the coatings are explained in Figure 6. The settlement of macrofouling organisms was also reduced on the panels coated with coatings (ICE15M and ECE15M) developed with the extracts of the bacterium *Bacillus subtilis*. MUT:M15. The number of barnacles recruited on the panel coated with control coating was 13±3.6 and this was reduced to 5.3±1.5 and 6±2 on the panels coated with ICE15M and ECE15M, respectively. One-way ANOVA showed significant variation (F=8.396, P<0.01) on the recruitment of barnacles between control and antifouling coatings coated panels. Similarly, there was a significant (One-way ANOVA: F=5.711, P<0.05) reduction on the settlement of tubeworms on the panels coated with bacterial extract incorporated antifouling coating. The number of tubeworms settled on the panel coated with control coating was 14.33±2 and this was reduced to 7.33±3.51 and 10±1.7 on the panels coated with ICE15M and ECE15M, respectively. Also, the density of gastropods on the panels coated with bacterial extracts was lower than the control panels. One-way ANOVA showed significant variation (F=5.166, P<0.04) on the settlement of gastropods between control and antifouling coatings coated panels. The

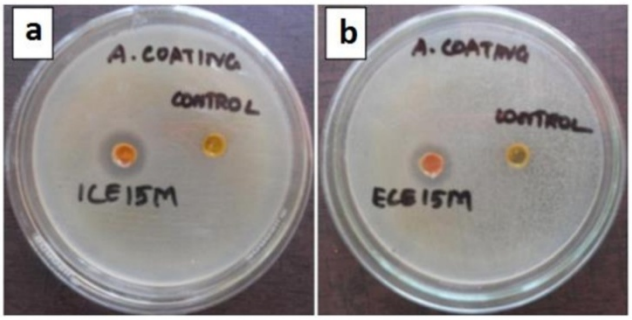

Fig. 4 — Bioactivity of coatings developed with bacterial extracts using agar over layer method. (a) Bioactivity of coating ICE15M developed with intracellular extract of the bacterium *B. subtilis*. (b) Bioactivity of coating ECE15M developed with extracellular extract of the bacterium *B. subtilis*.

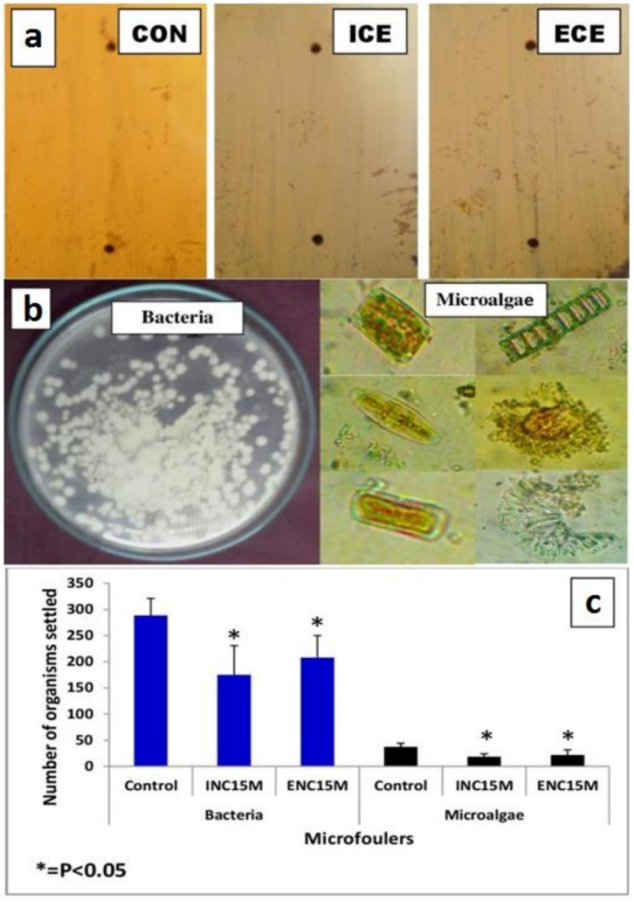

Fig. 5 — Assessment of anti-microfouling effect of coatings. (a) Panels submerged into the coastal water for a period of 5 days (b) Microbes settled on the panels (c) Plot showing the difference on the settlement of microfoulers between control and antifouling coating coated panels.

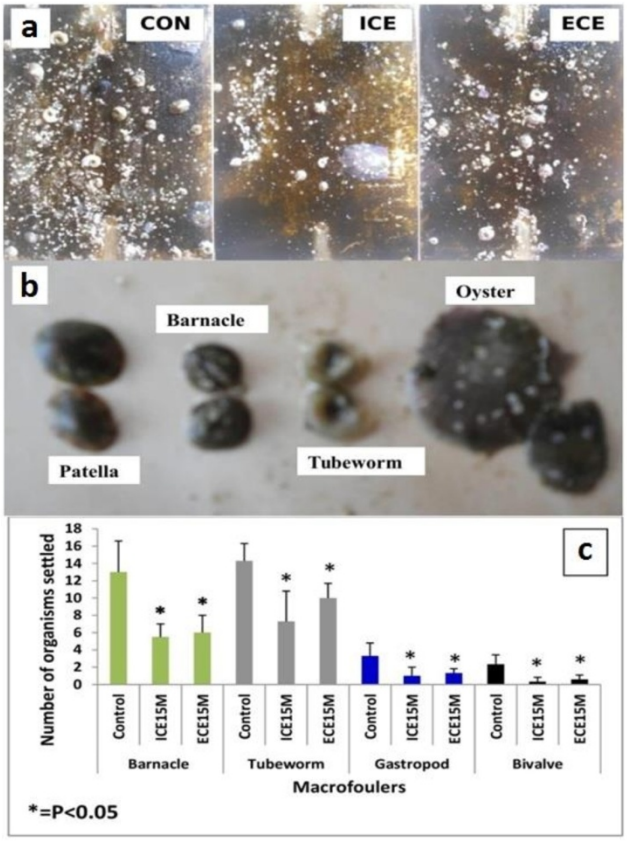

Fig. 6 — Assessment of anti-macrofouling effect of coatings (a) Panels submerged into the coastal water for a period of 100 days (b) Macroorganisms settled on the panels (c) Plot showing the difference on the settlement of microfoulers between control and antifouling coating coated panels.

bivalves settlement differed significantly between control and bacterial extract coated panels (F=5.375, P<0.04).

Biomass of fouling community recruited on the panels coated with ICE15M and ECE15M coatings showed substantial variation with the biomass of fouling community recruited on the panel coated with control coating (Fig. 6). The biomass of fouling community recruited on the panel coated with control coating was 86.39% and it was reduced to 54.05% (Student's *T*-test, t stat = 8.01, df = 3, *P*<0.002) and 70.44% Student's *T*-test, t stat = 5.54, df = 3, *P*<0.005) on the panels coated with ICE15M and ECE15M coatings (Fig. 7).

Isolation of bioactive fractions using thin-layer chromatography

The intracellular extract of the antagonistic bacterium *Bacillus subtilis*. MUT:M15 showed four spots with the Rf values of 0.43, 0.56, 0.80 and

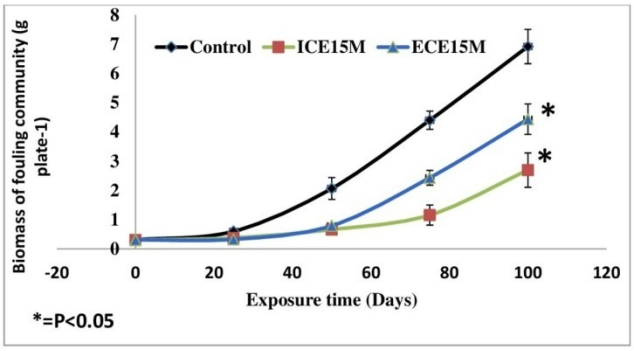

Fig. 7 — Recruitment of biofouling organisms obtained from the panels coated with control and antifouling coatings in the interval of 20 days.

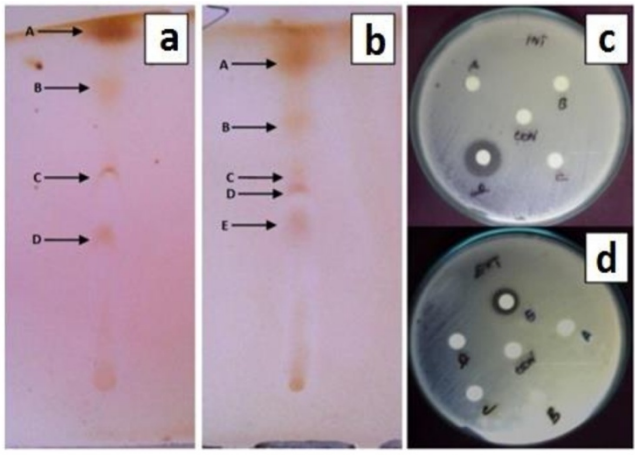

Fig. 8 — Isolation of bioactive fractions from the bacterial crude extract. (a) TLC spectrum of intracellular extract and (c) bioactivity of fractions obtained. (b) TLC spectrum of extracellular extract and (d) bioactivity of fractions obtained from the extracellular extract

0.92 cm (Fig. 8A). The spot with the Rf value of 0.43 showed activity against biofilm-forming bacterium *Alteromonas* sp (Fig. 8C). Whereas, the extracellular extract loaded silica gel plate showed five distinct spots on the silica gel plate with the Rf values of 0.47, 0.57, 0.65, 0.77 and 0.91 (Fig. 8B). Among them, the spot with the Rf value of 0.47 cm inhibited (12 mm) the growth of biofilm-forming bacterium, *Alteromonas* sp. (Fig. 8D).

FT-IR analysis of bioactive fractions

The FT-IR spectrum of the TLC resolved bioactive fraction isolated from both intracellular and extracellular extracts of the bacterium *Bacillus subtilis*. MUT:M15 showed the presence of major peaks at 1730-1700, 3400 and 1320-1210 cm^{-1}. The peaks between 1730-1700 represent C=O stretch and

peak near 3400 indicates the broad O-H, while the peaks between 1320-1210 cm-[1] represent a C-O stretch. Based on the peaks on the spectrum, it was concluded that the bioactive compounds present in the fractions were belonging to a carboxylic acid group (Figs 9A and 9B).

GC-MS analysis of bioactive fractions

GC–MS spectrum of the bioactive fraction isolated from the intracellular extract from the bacterium *Bacillus subtilis*. MUT:M15 showed a major peak at the retention time of 23.21 (Fig. 10A). This peak showed the presence of 13 phthalic acid derivatives. The details of the phthalate derivatives are given in Table 1. Similarly, a single major peak (Rt-13.87) appeared in the GC-MS spectrum (Fig. 10B) of the bioactive fraction isolated from the extracellular extract of the bacterium *Bacillus subtilis*. MUT:M15 showed the presence of 15 phthalate derivatives and their details are given in Table 2.

Discussion

Many studies have demonstrated the isolation and characterization of bioactive compounds produced by the bacteria from the marine environment[36,37]. For instance, Yu et al.[38] reported the production of a bacillamide C by the bacterium *Bacillus vallismortis.* In this study, the bacterium responsible for the production of antifouling compounds was identified as *B. subtilis*. Generally, *Bacillus* sp. is widely distributed in the marine environment and produces a variety of useful products including bioactive compounds[39-41]. For example, Satheesh *et al*.[30] isolated and identified two *Bacillus* sp. from the surface of the marine sponge which was responsible for the production of antifouling compounds. Likewise, bioactive (antibiofilm) compound producing bacterium *Bacillus licheniformis* associated with the surface of the sponge was isolated and identified by Sayem et al.[42]. Generally, it has been noted that *Bacillus* sp. are considered as one of the most bioactive compound producing bacterial species and have a great potential for biotechnological and biopharmaceutical applications[43,45].

In this study, bioactive bacterial extracts were incorporated into epoxy resin to develop eco-friendly

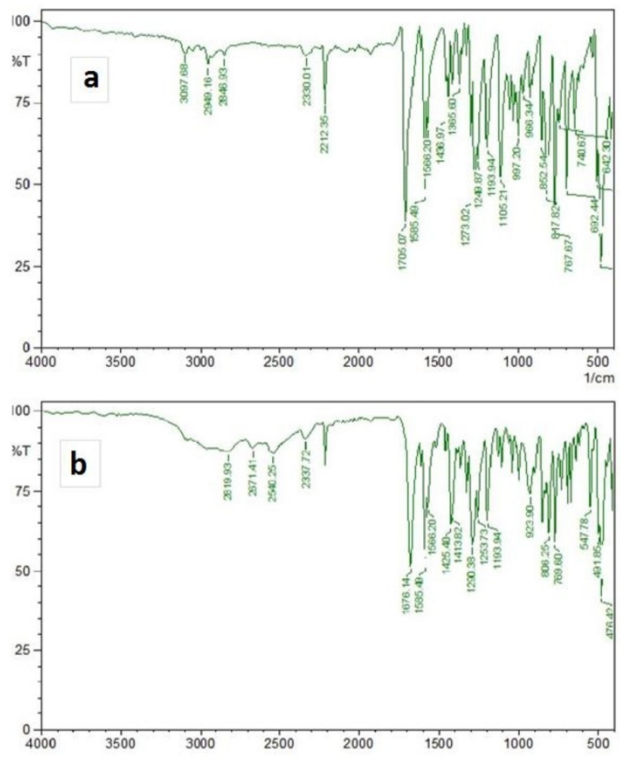

Fig. 9 — (a) FT-IR Spectrum of the bioactive fraction isolated from intracellular extract. (b) FT-IR Spectrum of the bioactive fraction isolated from extracellular extract.

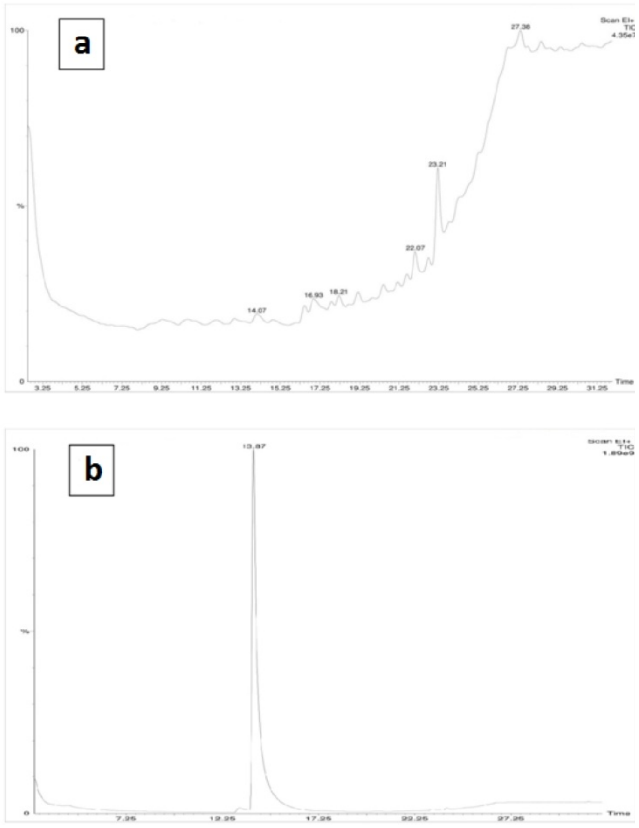

Fig. 10 — (a) GC-MS Spectrum of the bioactive fraction isolated from intracellular extract. (b) GC-MS Spectrum of the bioactive fraction isolated from extracellular extract.

Table 1 — Details of the phthalate derivatives present in the intracellular extract of the bacterium *Bacillus subtilis*.MUT:M15

S.No	Compound Name	M. Formula	M. Weight (g/mol)
1	1,2-Benzenedi carboxylic acid, Di isooctyl ester	$C_{24}H_{38}O_4$	390
2	1,2-Benzenedicarboxylic acid; Mono (2-Ethylhexyl) ester	$C_{16}H_{22}O_4$	278
3	Di-N-octyl phthalate	$C_{24}H_{38}O_4$	390
4	Decyl 2-Ethyl hexyl ester	$C_{18}H_{38}O_3S$	334
5	Bis (2-Ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390
6	2-Ethylhexyl tridecyl ester	$C_{29}H_{48}O_4$	460
7	6-Ethyloct-3-yl 2-ethylhexyl ester	$C_{26}H_{42}O_4$	418
8	2-Ethylhexyl undecyl ester	$C_{19}H_{40}O_3S$	348
9	2-Ethylhexyl pentadecyl ester	$C_{25}H_{48}O_4$	412
10	2-Ethylhexyl tetradecyl ester	$C_{30}H_{50}O_4$	474
11	4-Octyl tetradecyl ester	$C_{30}H_{51}NO_4$	489
13	3',8,8'-Trimethoxy-3-piperidyl-2,2'-binaphthalene-1,1',4,4'-tetrone	$C_{28}H_{25}NO_7$	487

Table 2 — Details of the phthalate derivatives present in the extracellular extract of the bacterium *Bacillus subtilis*.MUT:M15

S.No	Compound Name	M. Formula	M. Weight (g/mol)
1	Diethyl phthalate	$C_{12}H_{14}O_4$	222
2	Ethyl pent-2-en-4-yn-1-yl ester	$C_{15}H_{14}O_4$	258
3	Ethyl 3,4-dimethylphenyl ester	$C_{18}H_{18}O_4$	298
4	Ethyl 4- isoporpyl ester	$C_{19}H_{20}O_4$	312
5	4-Bromophenyl ethyl ester	$C_{16}H_{13}O_4\,Br$	348
6	4-Chloro-3-methylphenyl ethyl ester	$C_{17}H_{15}O_4CL$	318
8	2-Acethylphenyl ethyl ester	$C_{18}H_{16}O5_5$	312
9	Ethyl non-5-yn-3-yl ester	$C_{19}H_{24}O_4$	316
10	Cyclobutyl ethyl ester	$C_{14}H_{16}O_4$	248
11	3,4-Dichlorophenyl ethyl ester	$C_{16}H_{12}O_4CL_2$	338.
12	Ethyl 4-nitrophenyl ester	$C_{16}H_{13}O_6N$	315
13	3,5-Difluorophenyl ethyl ester	$C_{16}H_{12}F_2O_4$	306
14	Ethyl pentyl ester	$C_{16}H_{14}O_4$	270
15	Monoamide, n-ethyl-n-phenyl-, Ethyl ester	$C_{18}H_{19}O_3N$	297.

antifouling coatings. It has been reported that incorporation of natural products having repellent activity into the paint matrix could be used to control biofouling[46,47]. The less toxicity and biocidal property of the natural products would play significant role on the development of antifouling coatings. For example, Eguia and trueba[48] incorporated the surface associated bacterial extract into the paint for the development of innocuous antifouling coating. In this study, epoxy was used as the matrix due to its good mechanical and chemical properties and also it was widely used as surface coatings, adhesives, painting materials, laminates, encapsulants, polymer composites, and insulating materials for electronic devices, etc[49-51].

The bioactive nature of the bacterial extract may be changed when the biologically active compounds are incorporated into the paint (binder) and also these binders (paints) may not release the biocide into the surrounding medium by holding them itself. To find these things, in this study, an agar well diffusion method was used and the results showed that both (ICE15M and ECE15M) coatings prepared with bacterial extracts considerably inhibited the growth of biofilm-forming bacteria. From the result, it is understandable that there were no clear changes in the bioactivity of the bacterial extracts even after being incorporated into the paint and also the paint released the biocides into the agar. Jellali *et al*.[52] tested the bioactivity of coatings developed by natural rubber derived oligomers and found that all the coatings developed were found to be active against foulers. However, in this study, the leaching rate of the biocide (bacterial extract) from the paint was not considered, which must be important for possible further applications of natural product based antifouling coatings.

The result of the present study revealed that the panels coated with antifouling coatings considerably

reduced the settlement of marine bacteria and microalgae. Microorganisms such as bacteria, microalgae and fungi are the representatives of microfouling on a surface which will promote the settlement of following macrofoulers[53]. Such initial microfouling can be prevented by applying the paint with natural products having repellent activity[54,55]. The anti-microfouling effect of coatings in *in vitro* condition has been reported extensively in the literature. For example, Harder *et al*.[56] incorporated the bioactive compound into Phytagel and reported the inhibition of the settlement of cultivable and non-cultivable microbes (bacteria). Whereas, Satheesh *et al*.[30] studied the anti-microalgal effect of coating developed with sponge-associated bacterial extract and found that there was a reduction in the settlement of microalgae *Chlorella* sp. on the panel coated with antifouling coatings.

In addition, antifouling activity of coating was tested in the coastal waters only for a short period of 100 days. Results showed that the coatings considerably reduced the settlement of marine macrofouling organisms such as barnacles, tubeworms, bivalves and gastropods. All these marine invertebrates are reported as the major macrofouling organisms in the marine environment[57,58]. Besides, the assessment of fouling inhibition by antifouling coating also revealed that the panels coated with bacterial extract incorporated coatings considerably reduced the biomass of marine fouling organisms throughout the test period (100 days). Zhou *et al*.[59] conducted a similar study by incorporating a biologically active genistein (obtained from a plant) into resin based paint, and this paint coated panels were submerged in the sea over a period of three months. These authors found that the paint containing genistein considerably inhibited the settlement of marine biofouling organisms. Generally, antifouling studies were conducted only for a short period in the coastal waters or only limited to laboratory assays[60,61]. However, the 100-day study period used in this study is strong enough for getting sufficient information about an efficacious antifouling system, though long-term field trials are necessary to develop an antifouling coating for field applications.

The result of this study proved the hypothesis stated by Armstrong *et al*.[55] that the paint used to prevent initial microfouling (biofilm) would also prevent the settlement of subsequent macrofoulers. Supportively, Yang *et al*.[62] reported that a paint developed by incorporating the bioactive compound (Kalihinol) into Phytagel, prevented the colonization of bacteria and also the settlement of the larvae of *H. elegans*. The reason behind this is that a minimum microbial density is required for the settlement of larvae and the percentage of larval settlement is correlated with the bacterial density[63,64]. The microbial communities (biofilm) on the surfaces could vary depending on the bacterial species which would impose different influences on larval settlement[65]. Other studies have also supported that the bacterial composition may have profound effect on the larval settlement[66-68].

The bioactive fractions present in both intracellular and extracellular extracts of the antagonistic bacterium *B. subtilis* were isolated and identified as phthalate derivatives. There are many reports about the biosynthesis of phthalate derivatives by marine organisms and terrestrial plants[69-71]. Production of phthalate derivatives by microorganisms has also been documented extensively by many researchers[72-76]. For instance, Moushumi and Jayachandran[77] isolated the phthalate derivatives from the bacterium *Bacillus pumilus*. Although, the biosynthesis of phthalate derivatives by microorganisms has been reported extensively, their production process and physiological role have not yet been reported. Thus, the mode of action of phthalate derivatives on microbes must be studied.

Phthalate compounds are petrochemicals and have multiple applications in various sectors and used as plasticizers or solvents in a variety of industrial products and also in food handling and storage[78-80]. Moreover, it has been reported that phthalate derivatives are bioactive compounds and their bioactivities, such as antioxidant, antimicrobial, cytotoxic, anti-leukaemic, and antimutagenic activities have been analysed and documented by previous studies[81-86]. Based on the data obtained from this study, it is clear that the phthalate derivatives also have strong antifouling properties.

Conclusion

In this study, both antifouling coatings (ICE15M and ECE15M) developed with the intracellular and extracellular extract of the bacterium *Bacillus subtilis*. MUT:M15 were highly effective against marine biofouling organisms (both micro and macrofouling organisms) for a period of 100 days. As both coatings have considerable influence on antifouling,

further studies like leaching rate of the biocide present in the coating, durability and long-term field trials would certainly make a way for the development of an eco-friendly antifouling coating. Moreover, isolation characterization and application studies of each phthalate derivative produced by the bacterium *Bacillus subtilis*. MUT:M15 could lead to the synthesis of natural phthalates for antifouling applications.

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